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In re Application of:

Louis S. KUCERA *et al.*

Application No: 09/412,539

: Group Art Unit 1624

Filed: October 4, 1999

Title: LIPID ANALOGS FOR
TREATING VIRAL
INFECTIONS

: Examiner: Brenda Coleman

DECLARATION UNDER C.F.R. § 1.132

I hereby declare as follows:

1. I received a degree of Bachelor of Arts in Natural Science from St. John's University, Collegeville, Minnesota in 1957. I received a degree of Master of Science in Medical Microbiology from Creighton University, Omaha, Nebraska in 1959. I received a degree of Doctor of Philosophy in Medical Microbiology from University of Missouri, Columbia, Missouri in 1964.

2. I presently hold the position of Full Professor of Microbiology and Immunology at Wake Forest University School of Medicine, Winston-Salem, North Carolina, the assignee of the above-identified application, and Senior Vice-President/Founder of Kucera Pharmaceutical Company, Winston-Salem, North Carolina. I have been employed by Wake Forest University since 1970 and Kucera Pharmaceutical Company since 2001.

3. I am the author or co-author of over 130 publications, almost all of which pertain to antiviral agents or medical virology. I have also presented at numerous meetings, symposiums, workshops, and conferences and lectured at several universities.

4. My education, technical experience, professional activities, honors and awards, and list of publications are set forth in my *curriculum vitae* attached hereto as Exhibit A.

5. I am a co-inventor of the invention described and claimed in the above-identified application.

6. I am familiar with and understand the above-identified application ("Application"), including the pending claims and the outstanding rejections of the claims.

7. I and others have evaluated the activity of phospholipid-AZT conjugates against HIV. In our experiments, the lipid-AZT conjugates were synthesized by reacting 3-amino-1,2-propanediol with an acyl chloride to form an alkylamidopropanediol. The primary alcohol was protected as a trityl ether by reaction with trityl chloride and then the secondary alcohol was alkylated with an alkyl halide using sodium hydride as a base. After removing the trityl ether with p-toluenesulphonic acid, the hydroxy group was converted to a phosphatidic acid. The phosphatidic acid was coupled with AZT using dicyclohexylphosphodiimide as the condensing agent. The lipid-AZT conjugates were evaluated for cytotoxicity, anti-HIV-1 activity and selectivity in CEM-SS and PBL cells. Further details of the materials and methods are set forth in Kucera *et al.* "In vitro evaluation characterization of newly designed alkylamidophospholipid analogues as anti-human immunodeficiency virus type 1 agents," Antiviral Chemistry & Chemotherapy 9:157-165 (1998) ("Kucera *et al.*"), attached hereto as Exhibit B.

8. The INK-14 compound described in Table 1 of Kucera *et al.* is a compound within the genus of Formula III of the specification of the Application.

9. The INK-14 compound gave a high selectivity index ("SI") of >1250 against infectious HIV-1 replication in CEM-SS cells, as shown in Table 2 of Kucera *et al.*

10. The specification commencing on page 14, line 20 through page 15, line 8 of the Application describes that experimentation has demonstrated efficacy of the compounds of Formula III.

11. The data in Kucera *et al.* demonstrates that the INK-14 compound shows clinical efficacy of Formula III compounds against HIV.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: April 30, 2002

Louis S. Kuccra, Ph.D.
Louis S. Kuccra, Ph.D.

***In vitro* evaluation and characterization of newly designed alkylamidophospholipid analogues as anti-human immunodeficiency virus type 1 agents**

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Our laboratories first reported two novel classes of complex synthetic lipids, including alkylamidophosphocholines (PC lipid; CP-51) and alkylamidophosphate ester-linked lipid-AZT conjugates (lipid-AZT conjugates; CP-92), with selective and potent activity against human immunodeficiency virus type 1 (HIV-1). To extend these observations, we synthesized additional PC lipids and lipid-AZT conjugates (INK and INK-AZT conjugate) to evaluate their structure-activity relationships by testing for selectivity against infectious wild-type (wt) and drug-resistant HIV-1 replication, virus fusogenic activity and toxicity for mouse bone marrow cells. PC lipid compounds with medium chain lengths at positions 1 and 2 gave an improved selective index (SI). INK-3, with 12 and 8 carbons and INK-15, with 10 and 12 carbons were among the most selective when evaluated in CEM-SS cells. INK-14, a lipid-AZT conjugate where AZT replaced the choline in PC

lipid INK-3, gave the highest SI of >1250 against both infectious wt HIV-1 replication in CEM-SS cells and a clinical isolate in peripheral blood leukocytes. Notably, the PC lipid compounds INK-3 and INK-15, but not the lipid-AZT conjugate INK-14, were potent inhibitors of matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates. INK-3 also inhibited replication of HIV-2 and TIBO-resistant HIV-1, and inhibited HIV-1-mediated fusogenic activity by 78, 41 and 9% in a dose-dependent manner. The TC_{50} for mouse bone marrow cells was >100 µg/ml for INK-3 compared to 9.15–14.17 µg/ml for CP-51 and 0.142–0.259 µg/ml for AZT. These data suggest that optimum PC lipid compounds are significantly less toxic than AZT and have high potential as novel therapeutic agents for AIDS.

Keywords: alkylamidophosphocholine; phospholipid-nucleoside conjugate; HIV-1; AIDS

Introduction

Our laboratories first reported on two novel classes of complex synthetic lipids (alkylamidopropyl or alkylthioglycerol phosphocholines and phosphate ester-linked lipid-AZT conjugates) that have selective and potent activity against infectious human immunodeficiency virus (HIV) replication and pathogenesis *in vitro*. These compounds have a unique site of action involving alteration of HIV gp160/gp120 activity and production of defective virus particles (Kucera *et al.*, 1990a,b; Meyer *et al.*, 1991; Piantadosi *et al.*, 1991; Krugner-Higby *et al.*, 1995). A thiolipid-nucleoside conjugate (BM21-1290) has recently been introduced into Phase I/II tolerability and efficacy trials in ARC/AIDS patients by Boehringer Mannheim (Herrmann DBJ, Schleger C & Opitz HG; Antiretroviral

activity of the novel candidate anti-AIDS drug BM21.1290 in the Friend-virus leukemia system *in vivo*; *VIIth German AIDS Congress*, October 24–26 1997, Munich, Germany, Abstract V160; Herrmann DBJ, Opitz HG & Kucera LS; BM21-1290: evaluation of the antiretroviral activity of a new anti-AIDS drug *in vivo*. *IVth Conference on Retroviruses and Opportunistic Infections*, January 22–26 1997, Washington, DC, USA, Abstract p92). Among the reported alkylamidopropyl phosphocholine (PC lipid) analogues synthesized and tested for anti-HIV-1 activity, the most selective was compound CP-51, 1-octadecanamido-2-ethoxypropyl-3-phosphocholine (Kucera *et al.*, 1990a). In order to extend these observations, we synthesized a series of CP-51 analogues to

evaluate their structure-activity relationships and thus to optimize the PC lipid compound for future evaluation as a potential therapeutic agent for AIDS. Results of this investigation indicated that among the PC lipid compounds tested in CEM-SS cells, compounds INK-3 and INK-15 with intermediate hydrocarbon chain lengths of 12 and 10, respectively, at position 1, and of 8 and 12, respectively, at position 2, gave the highest selectivity index (SI) against infectious HIV-1 replication. Selected PC lipid compounds were active against AZT-resistant clinical isolates, TIBO-resistant HIV-1 and wild-type HIV-2, inhibited HIV-1 fusogenic activity, and were less toxic to mouse bone marrow cells compared to AZT. Based on these interesting results, optimum PC lipid compounds may have great potential as novel therapeutic agents for AIDS, either alone or in combination therapy.

Materials and Methods: Chemistry

All chemicals were used as provided by the supplier without further purification unless otherwise indicated. AZT was obtained from Boehringer Mannheim (Mannheim, Germany). Column chromatography was performed with silica gel 60 (230–400 or 70–230 mesh). Melting points were obtained on a Hoover Meltemp apparatus and are uncorrected. Proton NMR spectra were obtained on Bruker 300 MHz or Varian 400 MHz spectrometer as solutions in CDCl_3 with Me_4Si as an internal standard. The final compounds were, in general, hygroscopic solids. FAB mass spectra were run on a VG 70S mass spectrometer. Thin layer chromatography (TLC) of phosphocholines in CHCl_3 : MeOH : NH_4OH (70:35:7) and CHCl_3 : MeOH (2:1) gave a single spot, which gave a blue colour with a modified Dittmer–Lester reagent (Ryu & MacCoss, 1979). A representative synthetic procedure is given for INK-3; the remaining phosphocholines were prepared from the appropriate reagents using analogous procedures as those detailed below and in our previous papers (Piantadosi *et al.*, 1991; Meyer *et al.*, 1991; Morris-Natschke *et al.*, 1993). Complete high resolution mass spectra results are available as supplementary data from the Editor-in-Chief.

3-Dodecanamido-1,2-propanediol

3-Amino-1,2-propanediol (42 g, 0.47 mol) was dissolved in pyridine (150 ml) and DMF (200 ml). Dodecanoyl chloride (47 g, 0.46 mol) in 150 ml DMF was added dropwise. After 24 h at room temperature, the product was removed by filtration, washed with H_2O and recrystallized from MeOH , then from CHCl_3 . The amide was obtained in 28% yield (34.7 g, 0.013 mol, m.p. 94–96°C). ^1H NMR (CDCl_3): 0.90 (t, 3H, terminal CH_3), 1.25 [m, 16H (CH_2)₁₁], 1.6 (m, 2H, $\text{NHCOCH}_2\text{CH}_2$), 2.25 (t, 2H,

NHCOCH_2), 3.45 (m, 2H, CH_2NH), 3.6 (m, 2H, CH_2OH), 3.8 (m, 1H, CHOH), 5.8 (t, 1H, NH).

3-Dodecanamido-1-triphenylmethoxy-2-propanol

The above diol (5.5 g, 0.020 mol) was dissolved in dry pyridine (50 ml), then a solution of trityl chloride (5.6 g, 0.020 mol) in pyridine (50 ml) was added dropwise at 50°C. The reaction mixture was stirred at this temperature for 10 h and then cooled. The pyridine was removed under vacuum, and water (100 ml) was added to the solid residue. After filtration and washing, the crude product was partitioned between water and CHCl_3 . The water layer was extracted again with CHCl_3 and the organic layers combined and dried over anhydrous Na_2SO_4 . After removing solvent, the resulting solid was dissolved in hexane:ethyl acetate (3:1); 1 g of starting diol was filtered. The mother liquor was concentrated and chromatographed on silica gel with a discontinuous gradient of hexane:EtOAc to give 7 g (0.014 mol, 70% yield) of pure product. ^1H NMR (CDCl_3): 0.90 (t, 3H, terminal CH_3), 1.25 [m, 16H (CH_2)₁₁], 1.6 (m, 2H, $\text{NHCOCH}_2\text{CH}_2$), 2.2 (t, 2H, NHCOCH_2), 3.2–3.4 (overlapping m, 3H, CH_2NH and $\text{CH}_2\text{CH}_2\text{OTr}$), 3.55 (m, 1H, $\text{CH}_2\text{CH}_2\text{Tr}$), 3.9 (m, 1H, CHOH), 5.7 (t, 1H, NH), 7.3–7.5 (m, 15H, aromatic H).

3-Dodecanamido-2-octyloxy-1-triphenylmethoxypropane

The synthesized trityl ether (7.0 g, 0.013 mol) in 30 ml dry THF was added dropwise to an ice-cooled suspension of sodium hydride (1.0 g of 60% oil dispersion, 0.025 mol) in 100 ml THF under nitrogen. After warming to room temperature, heat was applied (60°C) for 1 h. 1-Bromooctane (2.9 g, 0.015 mol, neat) was added dropwise and heating continued for 10 h. After cooling, ice and then water were added slowly. The biphasic system was separated and the water layer extracted with EtOAc (3×50 ml). All organic fractions were combined and dried over anhydrous Na_2SO_4 . After removing solvent *in vacuo*, the residue was chromatographed with a gradient of hexane:EtOAc (100:0 to 6:1) to give 3.6 g (38% yield) of pure product. Impure product was rechromatographed using the same conditions to give an added 3.0 g (31% yield) of product as a viscous oil. ^1H NMR (CDCl_3): 0.85 (t, 6H, terminal CH_3), 1.25 [m, 26H (CH_2)₈ and (CH_2)₁₁], 1.45 (m, 4H, $\text{NHCOCH}_2\text{CH}_2$ and OCH_2CH_2), 2.2 (t, 2H, NHCOCH_2), 3.2–3.6 (overlapping m, 7H, CH_2CHCH_2 , OCH_2), 5.75 (t, 1H, NH), 7.3–7.5 (m, 15H, aromatic H).

3-Dodecanamido-2-octyloxy-1-propanol

Detritylation of the above compound (6.0 g, 9.5 mmol) was accomplished using *p*-toluenesulphonic acid (0.6 g, 3.1 mmol) in CHCl_3 : MeOH (60 ml:18 ml). The reaction

mixture was stirred at room temperature for 24 h then saturated NaHCO_3 solution was added and stirred for 30 min. The layers were separated and the CHCl_3 fraction dried over anhydrous Na_2SO_4 . After concentration *in vacuo*, the residue was purified twice by column chromatography using CHCl_3 : MeOH as eluent (100 : 0 and 15 : 1) to give 3.0 g (80% yield) of pure 3-dodecanamido-2-octyloxy-1-propanol (m.p. 42–43°C). ^1H NMR (CDCl_3): 0.9 (t, 6H, terminal CH_3), 1.25 [m, 26H (CH_2)₈ and (CH_2)₅], 1.6 (m, 4H, $\text{NHCOCH}_2\text{CH}_2$ and OCH_2CH_2), 2.2 (t, 2H, NHCOCH_2), 3.2–3.7 (overlapping m, 7H, CH_2CHCH_2 , OCH_2), 5.75 (t, 1H, NH).

3-Dodecanamido-2-octyloxypropyl 2-bromoethyl phosphate

3-Dodecanamido-2-octyloxy-1-propanol (1.0 g, 2.6 mmol) in 60 ml 2 : 1 anhydrous Et_2O : THF was cooled to 0°C. Pyridine (3.3 g) then 2-bromoethyl dichlorophosphate (2.4 g, 0.010 mol, prepared as described by Hansen *et al.*, 1992) were added dropwise. After warming to room temperature, the mixture was refluxed for 4 h, cooled and water (10 ml) added. After stirring for 30 min, the solvent was removed *in vacuo* and the residue dissolved in 100 ml CHCl_3 : MeOH (2 : 1). The solution was extracted with water and back-extracted with 2×50 ml 2 : 1 CHCl_3 : MeOH. The combined organic fractions were dried over anhydrous Na_2SO_4 , evaporated and chromatographed on silica gel using a gradient of CHCl_3 : MeOH (100 : 1 to 15 : 1). The desired bromoethylphosphate (633 mg, 1.1 mmol) was obtained in 43% yield. ^1H NMR (CDCl_3): 0.85 (t, 6H, terminal CH_3), 1.25 [m, 26H (CH_2)₈ and (CH_2)₅], 1.55 (m, 4H, $\text{NHCOCH}_2\text{CH}_2$ and OCH_2CH_2), 2.3 (t, 2H, COCH_2), 3.4–3.7 (overlapping m, 7H, CHCH_2NHCO , CH_2Br , OCH_2), 3.85 and 4.25 (two m, 4H, $\text{CH}_2\text{OPO}_3\text{CH}_2$), 6.8 (m, 1H, NH).

3-Dodecanamido-2-octyloxypropyl phosphocholine (INK-3)

The above phosphate ester (633 mg, 1.1 mmol) in 52 ml CHCl_3 : isopropanol : DMF (5 : 3 : 5) was reacted with aqueous Me_3N (7.6 ml) at 65°C for 5 h. After cooling, Ag_2CO_3 (380 mg) was added and the heat reapplied for 1 h. After filtering the precipitated AgBr , the solvent was removed *in vacuo* and the residue purified by chromatography on silica gel using CHCl_3 : MeOH (10 : 1 to 2 : 1) followed by CHCl_3 : MeOH : NH_4OH (75 : 25 : 5) to give 275 mg pure (46% yield) and 140 mg impure phosphocholine. FAB MS showed a $[\text{MH}]^+$ ion at 551.418345 ($\text{C}_{38}\text{H}_{70}\text{N}_2\text{O}_6\text{P}$, 1.0 p.p.m.). ^1H NMR (CDCl_3): 0.87 (t, 6H, terminal CH_3), 1.25 [m, 26H (CH_2)₈ and (CH_2)₅], 1.5 (m, 4H, $\text{NHCOCH}_2\text{CH}_2$, OCH_2CH_2), 2.2 (t, 2H, COCH_2), 3.3 [s, 9H, $\text{N}(\text{CH}_3)_3$], 3.3–3.8 (overlapping m, 5H, $\text{CH}_2\text{CHOCH}_2$) (3.8–3.9, overlapping m, 4H, CH_2OP ,

CH_2NMe_3), 4.4 (m, 2H, PO_3CH_2), 7.0 (m, 1H, NH).

3-Dodecanamido-2-octyloxypropyl diphenyl phosphate

Diphenylchlorophosphate (0.4 ml, 1.7 mmol) in 10 ml diethyl ether was cooled to 4°C under nitrogen. 3-Dodecanamido-2-octyloxy-1-propanol (500 mg, 1.3 mmol) in 8 ml pyridine and 10 ml Et_2O was added dropwise. The solution was warmed to room temperature, then heated to around 52°C for 4 h and cooled to room temperature, diluted with 30 ml Et_2O , washed with 15 ml portions of water, 0.5 M HCl and water. The organic layer was dried over anhydrous Na_2SO_4 , filtered and concentrated *in vacuo* to an oil. Chromatography with a gradient of hexane : EtOAc (10 : 1 to 3 : 1) gave 556 mg (0.9 mmol, 69%) of pure product. ^1H NMR (CDCl_3): 0.9 (t, 6H, terminal CH_3), 1.25 [m, 26H (CH_2)₈ and (CH_2)₅], 1.55 (m, 4H, $\text{NHCOCH}_2\text{CH}_2$ and OCH_2CH_2), 2.3 (t, 2H, COCH_2), 3.3–3.7 (overlapping m, 5H, OCH_2 , CHCH_2NHCO), 4.25 (m, 2H, CH_2OP), 5.9 (m, 1H, NH), 7.2–7.8 (m, 10H, aromatic H).

3-Dodecanamido-2-octyloxypropyl phosphatidic acid

PtO_2 (81 mg) was placed in a Parr hydrogenation bottle. 3-Dodecanamido-2-octyloxypropyl diphenyl phosphate (589 mg, 1.0 mmol) in 118 ml EtOH was then added. After hydrogenation at 158.5 kPa (23 p.s.i.) for 4.5 h, the reaction mixture was filtered and checked for completion by TLC. To ensure complete conversion to the phosphatidic acid, hydrogenation was continued for another 6 h using 120 mg of fresh catalyst. The reaction mixture was then filtered through Celite and the EtOH removed *in vacuo*. The residue was chromatographed on silica gel using 4 : 1 CHCl_3 : MeOH as eluent to obtain 233 mg (0.54 mmol, 54% yield) of pure phosphatidic acid. ^1H NMR (CDCl_3): 0.9 (t, 6H, terminal CH_3), 1.25 [m, 26H (CH_2)₈ and (CH_2)₅], 1.55 (m, 4H, $\text{NHCOCH}_2\text{CH}_2$ and OCH_2CH_2), 2.3 (t, 2H, COCH_2), 3.3–3.7 (overlapping m, 5H, OCH_2 , CHCH_2NHCO), 4.0 (m, 2H, CH_2OP), 6.8 (m, 1H, NH).

3'-Azido-3'-deoxy-5'-(3-dodecanamido-2-octyloxypropyl)-phosphothymidine (INK-14)

The procedure of Piantadosi *et al.* (1991) was followed to synthesize INK-14 in 22% yield from the above phosphatidic acid and AZT. FAB MS showed a $[\text{MH}+\text{Na}]^+$ ion at 737.403800 ($\text{C}_{38}\text{H}_{70}\text{N}_6\text{O}_6\text{PNa}$, -8.0 p.p.m.). ^1H NMR (CDCl_3): 0.9 (t, 6H, terminal CH_3), 1.25 [m, 26H (CH_2)₈ and (CH_2)₅], 1.55 (m, 4H, $\text{NHCOCH}_2\text{CH}_2$ and OCH_2CH_2), 1.8 (s, 3H, thymine CH_3), 2.2 (t, 2H, COCH_2), 2.3–2.55 (m, 2H, 2'- CH_2), 3.3–3.6 (overlapping m, 5H, OCH_2 , CHCH_2NHCO), 3.8–4.2 (overlapping m, 5H, $\text{CH}_2\text{OPO}_3\text{CH}_2$, 4'-CH), 4.5 (1H, 3'-CH), 6.15 (m, 1'-CH), 7.4 (m, 1H, thymine CH).

Materials and Methods: Virology

Virus stocks

HIV-1 (strain IIB) was propagated in H9IIB cells as previously described (Krugner-Higby *et al.*, 1995). Stocks of HIV-2 from R Weiss (Institute of Cancer Research, London, UK), TIBO-resistant HIV-1 (RT mutant at codons 103 and 181) from E Emini (Merck, Sharp & Dohme Research Laboratories, West Point, Pa., USA) and AZT-resistant HIV-1 isolates 1073 (RT mutant at codons 70 and 215) and 1074 (RT mutant at codons 70 and 215), and matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates G-762 (wild-type), G-691 (RT mutant) and H112-2 (wild-type), G-910 (RT mutant at codon 215) from D Richman (University of California, La Jolla, Calif., USA) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS Program, NIH, Bethesda, Md., USA). Stocks of viruses were propagated in acutely infected CEM-SS cells as previously described (Krugner-Higby *et al.*, 1995).

Cell cultures

All primary peripheral blood leukocytes (PBL) and cell lines were incubated and maintained in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), 2 mM L-glutamine and 20% fetal bovine serum (growth medium) as previously described (Krugner-Higby *et al.*, 1995). PBL were obtained from HIV-1-seronegative male and female personnel in the laboratory. Whole blood was separated into PBL using Isolymph (Gallard-Schlesinger Industries, Carle Place, N.Y., USA) according to a protocol supplied by the manufacturer. The separated PBL were activated for 3 days in the presence of phytohaemagglutinin (PHA; 5 µg/ml in growth medium), washed in growth medium, treated with polybrene (5 µg/ml in growth medium) and infected with HIV-1 in the presence of polybrene (1 µg/ml in growth medium). After a 2 h virus attachment, the cells were washed with growth medium to remove unattached HIV-1 and incubated (1×10^6 cells/ml of growth medium) for 96 h with or without added test compounds. Supernatant medium was harvested to measure virus replication by reverse transcriptase (RT) activity (Krugner-Higby *et al.*, 1995).

TC₅₀ determinations

To measure the cytotoxicity in CEM-SS cells, test compounds were dissolved in 95% ethanol, methanol: chloroform (1:1) or RPMI 1640 plus 20% FBS growth medium, diluted in log₁₀ or 0.5 log₁₀ series in growth medium and tested for cytotoxicity in triplicate wells containing 10 000 CEM-SS cells/well of a 96-well plate. A viable cell count was made prior to the test to ensure more

than 90% cell viability. Log phase cells were treated with test compound for 48 h at 37°C (approximately 2.5 cell generations in untreated control cultures) and pulse-labelled with 1 µCi [³H]TdR (sp. act. 20 Ci/mmol) for 6 h before harvesting the cells using a multichannel cell harvester to measure total DNA synthesis in the presence or absence of compound. To measure cytotoxicity in PBL, the cells were activated with PHA (5 µg/ml) for 72 h, washed to remove the PHA and 20 000 activated PBL cells/well were cultured for 96 h in growth medium with or without test compound as described for CEM-SS cells. The treated PBL cells were labelled with 0.1 µCi [³H]TdR (sp. act. 20 Ci/mmol) during the final 24 h culture period before harvesting. From the data, a TC₅₀ for cytotoxicity was calculated as previously described by Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Piantadosi *et al.* (1991).

Syncytium plaque assay

The syncytium plaque assay for infectious virus multiplication was done in the presence or absence of PC lipid or lipid-AZT conjugate as previously described by us (Kucera *et al.*, 1990a). The syncytium plaques were counted on day 5 or 6 post-infection and treatment and the percentage inhibition and effective concentration₅₀ (EC₅₀) were calculated by the method of Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Piantadosi *et al.* (1991).

Fusogenic assay

Persistently HIV-1-infected H9IIB cells were treated with compound for 96 h and cocultured with uninfected CD4⁺ CEM-SS cells (ratio 1:100) in the presence of added compound for 48 h. The number of fusogenic cells was counted relative to untreated control cells to determine the percentage inhibition of fusogenic cells. The EC₅₀ values were calculated by the method cited by Piantadosi *et al.* (1991).

Colony forming units-granulocyte macrophage (CFU-GM) assay

Mouse bone marrow granulocyte/macrophage cells were harvested and suspended in soft agar medium with or without added test compound (Herrmann DBJ, Kucera LS, Zilch H, Mertens A & Opitz HG; BM21.1290: in-vitro evaluation of a potential new anti-AIDS compound; *XIth International Conference on AIDS*, July 7-12 1996, Vancouver, Canada, Abstract p64). After incubation, the number of cell colonies was counted to determine the percentage inhibition of colony formation relative to a mock (PBS)-treated control. The 50% toxic concentration (TC₅₀) was calculated by the method cited by Piantadosi *et al.* (1991).

Results

Chemistry


Phospholipid and lipid-AZT conjugates were synthesized in our laboratories as previously described (Piantadosi *et al.*, 1991). Briefly, 3-amino-1,2-propanediol was reacted with an acyl chloride to form an alkylamidopropanediol. The primary alcohol was protected as the trityl ether by reaction with trityl chloride, then the secondary alcohol was alkylated with the appropriate alkyl halide using sodium hydride as the base. After removing the trityl ether with *p*-

toluenesulphonic acid, the hydroxy group was converted either to a phosphocholine [reaction first with bromoethyl phosphodichloridate (Hansen *et al.*, 1982) then with aqueous trimethyl amine] or, in the case of INK-14, to the phosphatidic acid (reaction with diphenyl chlorophosphate then catalytic hydrogenolysis over PtO_2). The phosphatidic acid was coupled with AZT using dicyclohexylphosphodiimide as the condensing agent.

Evaluation of compounds for cytotoxicity and anti-HIV-1 activity

To optimize the activity and/or SI, PC lipid and lipid-AZT conjugate compounds varying in hydrocarbon chain lengths at position 1 or 2 were synthesized (Table 1) and evaluated for cytotoxicity, anti-HIV-1 activity and SI in CEM-SS and PBL cells. Results of these experiments (Table 2) using CEM-SS cells indicated that the TC_{50} ranged from 27.6 (INK-2) to $>100 \mu\text{M}$ (INK-13 and -14). The EC_{50} ranged from <0.08 (INK-14) to $1.02 \mu\text{M}$ (INK-8). The SI ranged from 53.2 (INK-8) to >1250 (INK-14) (Table 2). Compounds with a 12 or 10 carbon chain length at position 1, an 8 or 12 carbon chain length at position 2, and phosphocholine at position 3 of the three carbon backbone (INK-3 or INK-15) gave the highest SI (298.9 and 389.0, respectively) among the PC lipid compounds. INK-14, which has the same hydrocarbon chain lengths at position 1 and position 2 as INK-3 but is linked at position 3 through a phosphate ester bond to AZT in place of phosphocholine (see Table 1), gave the highest SI (>1250)

Table 1. Chemical structures of synthetic phospholipids

Compound				
	A	B	C	D*
CP-51	NHCO	16	1	PC
INK-1	NHCO	10	11	PC
INK-2	NHCO	10	9	PC
INK-3	NHCO	10	7	PC
INK-4	O	11	9	PC
INK-6	NHCO	10	5	PC
INK-7	NHCO	16	7	PC
INK-8	NHCO	10	7	$\text{OPO}_3(\text{CH}_2)_8\text{N}(\text{CH}_3)_3$
INK-13	NHCO	8	7	PC
INK-14	NHCO	10	7	OPO_3AZT
INK-15	NHCO	8	11	PC

*PC, $\text{OPO}_3\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_3$.

Table 2. Evaluation of PC lipid and lipid-AZT conjugate compounds for cell cytotoxicity, anti-HIV-1 activity and SI in CEM-SS and PBL cells*

Compound	$\text{TC}_{50} (\mu\text{M})^\dagger$		$\text{EC}_{50} (\mu\text{M})^\dagger$		SI [‡]	
	CEM-SS	PBL	CEM-SS	PBL	CEM-SS	PBL
INK-1	30.3±8.9	43.7	0.47±0.2	1.25	64.5	34.9
INK-2	27.6±5.7	48.4	0.15±0.1	>3	184.0	<16.1
INK-3	56.8±22.4	>50	0.19±0.1	>3	298.9	~ 16.7
INK-4	75.7±21.7	48.2	0.64±0.4	>3	118.3	<16.1
INK-6	40.2±12.8	>50	0.21±0.1	0.63	191.4	>79.4
INK-7	36.0	31.6	0.67	1.5	53.7	21.0
INK-8	54.3±14.3	>50	1.02±0.3	>3	53.2	~ 16.7
INK-13	$>100\pm 0.0$	>50	0.77±0.7	2.5	>129.9	>20.0
INK-14	$>100\pm 0.0$	>50	$<0.08\pm 0.08$	0.62	>1250	>80.6
INK-15	38.9±2.9	42.1	0.10±0.06	>3	389.0	<14.0
CP-51§	14.3±2.3	ND	0.11±0.06	ND	130	—
AZTS	3.7±1.6	ND	0.009±0.008	ND	411	—

*The TC_{50} (cytotoxicity) and EC_{50} (anti-HIV-1 activity) data from CEM-SS cells represent averages \pm SD of 2–4 independent experiments per compound except for compound INK 7, which was tested once. Data from PBL cells were from a single independent experiment. To determine the TC_{50} , five serial concentrations of each compound were assayed in triplicate for ^3H ThdR incorporation into total DNA. To determine the EC_{50} , three to five serial concentrations of each compound were assayed in duplicate. The syncytial plaque assay was used with CEM-SS of HIV-1 (strain H112-2) as cited in Kucera *et al.* (1990a).

$^\dagger\text{TC}_{50}$ and EC_{50} values were calculated by the method of Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Piantadosi *et al.* (1991).

‡ SI is the ratio $\text{TC}_{50}/\text{EC}_{50}$.

§Data from Piantadosi *et al.* (1991).

Table 3. Evaluation of PC lipids INK-3 and CP-51 and AZT against TIBO-resistant, AZT-resistant HIV-1 and wild-type HIV-2 measured by syncytial plaque assay

Compound	EC ₅₀ (μM)*					
	TIBO	1073	1074	G910	G691	HIV-2
INK-3	0.11	0.90	0.19	0.20	0.12	0.21
CP-51	0.10	0.19	0.20	0.64	0.33	0.32
AZT	ND	0.16	0.03	>2.5	>2.5	ND

*EC₅₀ values were calculated by the method of Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Plantadosi *et al.* (1991).

Table 4. Evaluation of INK compounds for activity against matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates

Compound	EC ₅₀ (μM)		Fold increase res./sen.
	AZT-sensitive strain	AZT-resistant strain	
	G-762	G-691	
INK-14	0.10	2.34	23.4
INK-15	0.06	0.08	1.3
AZT	0.003	2.20	673.3
	H112-2	G-910	
INK-14	0.01	4.69	469
INK-15	0.07	0.02	<1
AZT	0.004	0.30	75

Assays were carried out using CEM-SS cells and the syncytial plaque assay.

Abbreviations: res: resistant; sen: sensitive

among all the compounds evaluated. In PBL cells, the TC₅₀ values were comparable to those from CEM-SS cells. However, the EC₅₀ values were consistently higher in PBL than in CEM-SS cells (Table 2). These results were most likely due to differences in sensitivity of prototype HIV-1 (strain H9IIIB) and a clinical isolate (strain H112-2) to the test compounds.

Inhibition of HIV-2 and TIBO- and AZT-resistant HIV-1 strains as measured by syncytial plaque assay

Since selected PC lipid compounds (for example INK-3 and CP-51) have potent activity against prototype strains of HIV-1, we extended evaluation of their activity against HIV-2 and TIBO- and AZT-resistant HIV-1 clinical isolates. Results (Table 3) indicated that both INK-3 and CP-51 gave dose-dependent activity against HIV-2, with EC₅₀ values of 0.21 and 0.32 μM and against TIBO-resistant HIV-1 with EC₅₀ values of 0.11 and 0.10 μM, respectively. The EC₅₀ values for INK-3 and CP-51 against AZT-resistant HIV-1 clinical isolates (1073, 1074, G910, G691) ranged from 0.12 to 0.90 μM and 0.19 to 0.64 μM, respectively (Table 3). The EC₅₀ values for AZT against

the same AZT-resistant HIV-1 clinical isolates ranged from 0.03 to >2.5 μM (Table 3). Note that the EC₅₀ for AZT against a sensitive prototype HIV-1 strain was 0.009 μM (Table 2). Other results indicated that the PC lipid INK-15 had no significant fold increase in EC₅₀ between the matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates (Table 4). However, the lipid-AZT conjugate INK-14 and AZT alone showed a significant fold increase in the EC₅₀ ratio between the AZT-sensitive and AZT-resistant HIV-1 clinical isolates of 23.4, 469 and 673.3, 75, respectively. In summary, the PC lipids INK-3 and CP-51 have potent activity against HIV-2 and TIBO- and AZT-resistant HIV-1. There is no apparent cross-resistance between PC lipid and TIBO or AZT compounds. In contrast, the lipid-AZT conjugate (INK-14) did show some cross-resistance with AZT.

Inhibition of fusogenic activity between persistently HIV-1-infected H9IIIB cells and uninfected CD4⁺ CEM-SS cells

Published data from our laboratories indicated that CP-51 significantly inhibited HIV-1-induced fusogenic activity (Krugner-Higby *et al.*, 1995). In similar experiments (Table 5), results indicated that INK-3 also has significant dose-dependent activity against HIV-1-induced fusogenic activity. The EC₅₀ values were 0.08, 0.18 and >1.0 μM for INK-2, -3, and -1, respectively (Table 5). These results suggest that selected PC lipid compounds active against infectious HIV production (Table 2) also inhibited virus-induced fusogenic activity.

Toxicity against mouse bone marrow cells as compared to AZT

Two independent experiments were designed to compare the relative toxicity of CP-51 and selected INK compounds with AZT. Results of these studies indicated that INK-2, -3 and -6 had a TC₅₀ value of >100 μg/ml (Table 6). In two experiments, the TC₅₀ values for CP-51 were 9.15 and 14.17 μg/ml compared to 0.142 and 0.259 μg/ml for AZT (Table 6). These data are interpreted to suggest that selected PC lipid compounds are significantly less cytotoxic than AZT to mouse bone marrow cells.

Discussion

Most of our previously synthesized alkylamidopropyl phosphocholines and alkoxy- and alkylthioglycerol phosphocholines (PC lipids) were analogues of phosphatidylcholine and contained a long hydrocarbon chain length (C16-C18) at position 1 and a short chain length (C1-C2) at position 2 of the three carbon backbone. Many of these compounds exhibited potent anti-HIV-1 activity. The most promising analogue we have reported to date

Table 5. Effect of PC lipid compounds on fusogenic activity between persistently HIV-1-infected H9IIIB cells and uninfected CD4⁺ CEM-SS cells

Compound	Concentration (μ M)	Inhibition of fused cells (%)	IC ₅₀ (μ M)
INK-1	1.0	18	>1.0
	0.1	27	
	0.01	17	
INK-2	1.0	86	0.08
	0.1	53	
	0.01	17	
INK-3	1.0	78	0.18
	0.1	41	
	0.01	9	

Persistently HIV-1-infected H9IIIB cells were treated with compound for 96 h and cocultured with uninfected CD4⁺ CEM-SS cells (ratio 1:100) in the presence of added compound for 48 h. The number of fused cells was counted relative to untreated control cells to determine the percentage inhibition of fusion and the IC₅₀. The IC₅₀ values were calculated by the method of Chou and coworkers (Chou & Talalay, 1987; Johnson *et al.*, 1989) as cited by Plantadosi *et al.* (1991).

Table 6. *In vitro* cytotoxicity of PC lipid compounds in CFU-GM assays

Compound	TC ₅₀ (μ g/ml)	
	Experiment 1	Experiment 2
CP-51	9.15	14.17
INK-2	>100	>100
INK-3	>100	>100
INK-6	>100	>100
AZT	0.142	0.259

In control cultures treated with PBS there were 86 and 54 c.f.u./plate in Experiments 1 and 2, respectively.

(Kucera *et al.*, 1990a) was 1-octadecanamido-2-ethoxypropyl-3-phosphocholine (CP-51). This amidoalkyl derivative exhibited an EC₅₀ of 0.11 μ M and a SI of 130 (Table 2). Accordingly, we chose this analogue as our lead compound and proceeded to modify its structure to optimize its anti-HIV-1 inhibitory activity and/or SI. In addition, we wanted to ascertain whether a long hydrocarbon chain length at position 1 was essential for optimum anti-HIV-1 selectivity.

As seen from data in Table 2, numerous synthetic CP-51 analogues including INK-2, INK-3, INK-6 and INK-15 exhibited a higher SI than CP-51. The SI values for these analogues in CEM-SS cells were 184.0, 298.9, 191.4 and 389.0, respectively, whereas their EC₅₀ values were comparable to that of CP-51. These results demonstrated that a long hydrocarbon chain length of 16–18 carbons is not essential for optimum selectivity in this series of PC compounds. Notably, data presented in Table 2 suggested that a shorter hydrocarbon chain length (Table 1) at position 1 (10 or 12 carbons) and a longer chain at

position 2 (12 or 8 carbons) as seen in INK-15 and INK-3, respectively, improved the SI up to threefold (with a concomitant decrease in cytotoxicity) compared with the 18 and 2 hydrocarbon chain lengths in CP-51.

Previously, we synthesized a series of alkyletherglycerol phosphocholines (Meyer *et al.*, 1991). Like the alkylamido phosphocholine CP-51, these alkylether compounds had a long hydrocarbon chain (C16–C18, oxy- or thioether) at position 1 and a short chain (methoxy or ethoxy) at position 2. The EC₅₀ values for these alkylether phosphocholines ranged from 0.3 to 1.4 μ M and their SI were lower (4 to 69) compared to that of CP-51 (130). In view of the above data with the INK compounds, it was of interest to synthesize an alkylether analogue of INK-2 to determine whether the SI could be similarly improved in this series. This alkylether analogue, INK-4, with oxyether hydrocarbon chain lengths of 12 and 10 at the 1 and 2 positions, respectively, exhibited an EC₅₀ of 0.64 μ M, a TC₅₀ of 75.7 μ M, and a SI of 118.3. Notably, the TC₅₀ value is significantly different from that of any previously reported alkylether phosphocholine (3 to 19 μ M; Meyer *et al.*, 1991), again demonstrating that such hydrocarbon chain length modifications will lead to a less cytotoxic compound. In summary, these data indicated that hydrocarbon chain lengths in the range of 10 to 12 at position 1 and 8 to 12 at position 2 should result in compounds with less cytotoxicity than those with 16 to 18 hydrocarbon chain lengths at position 1 and 1 to 2 at position 2.

Also, the TC₅₀ values from PBL cells were comparable to those from CEM-SS cells (Table 2). INK-13 and INK-14, with 8 and 10 carbons at position 1 and 7 carbons at position 2, respectively, were the least cytotoxic (TC₅₀ \geq 100 μ M) compounds in the series. At the present time, no specific experiments have been done to determine why INK-13 and INK-14 were the least cytotoxic in the series of compounds evaluated. In the case of the conjugate INK-14, the hypothesis is that the lipid component acts as a carrier for AZT. The lipid anchors the conjugate into the cell membrane and is metabolized to slowly release lower concentrations of AZT into cells compared to AZT treatment alone. The end result is reduced toxicity from AZT. Evidence in support of this hypothesis is the lower TC₅₀ and higher SI for INK-14 compared to AZT (Table 2).

In the case of the phospholipid-AZT conjugates, INK-14 [3'-azido-3'-deoxy-5'-(3-dodecylamido-2-octoxypropyl)-phosphothymidine] and CP-92 [3'-azido-3'-deoxy-5'-(3-octadecylamido-2-ethoxypropyl)-phosphothymidine] had comparable SI values [$>$ 1250 (Table 2) and 1793 (Plantadosi *et al.*, 1991), respectively]. Both compounds had a higher SI than AZT alone (411, Table 2) and were three- to fourfold more selective than AZT. In addition, the cytotoxicity was lower; the TC₅₀ for INK-14 was $>$ 100 μ M in comparison to 3.7 μ M for AZT.

The effects of lengthening the linkage between the phosphate ester and the quaternary ammonium functionality were also studied through the synthesis of INK-8, with three carbons separating the phosphate ester and the quaternary nitrogen. This modification increased the EC_{50} and decreased the SI values (Table 2). At the present time, it is difficult to draw any definitive conclusion relating to such modification.

Based on these promising *in vitro* data, selected PC lipid and lipid-AZT conjugates were further evaluated. The results indicated that CP-51 and INK-3 also were active against HIV-2 and TIBO-resistant HIV-1 (Table 3). We also examined the effects of CP-51 and INK-3 against AZT-resistant HIV-1 clinical isolates (1073, 1074, G910, G691). As seen from the data in Table 3, both CP-51 and INK-3 were markedly active against the AZT-resistant mutants. Both the nucleoside analogue AZT and the non-nucleoside TIBO analogue R82150 (nevirapine; White *et al.*, 1991) are known to inhibit HIV-1 RT (De Clercq, 1995). However, unlike AZT, which can also inhibit HIV-2 RT activity, the non-nucleoside RT inhibitors do not inhibit HIV-2 RT activity. This difference in antiviral spectrum may be due to the non-nucleoside analogue binding at an allosteric site of RT, in contrast to the active site like AZT (De Clercq, 1995). From our present data, PC lipids are active against both TIBO- and AZT-resistant strains of HIV-1, suggesting that PC lipids do not target the same site(s) in the HIV replication cycle as compared to TIBO derivatives and AZT.

Using matched pairs of AZT-sensitive and AZT-resistant HIV-1 clinical isolates, results indicated that each pair of isolates tested was similarly inhibited by the PC lipid INK-15 (Table 4). These data are interpreted to suggest that PC lipids do not show cross-resistance with AZT. In contrast, these same pairs of clinical isolates did show increased resistance to the conjugate compound INK-14 and to AZT alone (Table 4), suggesting that the most active anti-HIV-1 component in the lipid-AZT conjugate compound is probably AZT.

Results in Table 5 indicate that PC lipids were also active in inhibiting fusogenic activity of HIV-1-infected cells with uninfected cells. Recent published evidence by other investigators (Feng *et al.*, 1997) indicated that HIV-1 enters cells via a co-receptor designated fusin (CXCR-4), a member of the G protein-coupled chemokine receptor family with seven member-spanning domains. McKnight *et al.* (1997) reported that a monoclonal antibody to fusin can block cell-to-cell fusion and cell-free virus infection of fusin-positive CD4⁺ cells. Lipid analogues are known to accumulate at the cell plasma membrane (Storme *et al.*, 1985; van Blitterswijk *et al.*, 1987) and they serve as potent inhibitors of HIV-1-induced cell fusion (Krugner-Higby *et al.*, 1995). This inhibition was correlated with inhibition of

gp160/gp120-specific monoclonal antibody with HIV-1 gp160/gp120 reactivity on the surface of HIV-1-infected and treated cells (Krugner-Higby *et al.*, 1995). It can be speculated that the presence of lipid analogues could cause steric hindrance of the CD4⁺ receptor and fusin co-receptor in cell-to-cell fusion.

Published evidence indicates that the major antiviral effect of recombinant interferon α (rIFN- α) operates in the stages of virus assembly of gp120 and release (Hansen *et al.*, 1992; Smith *et al.*, 1991; Willey *et al.*, 1988). Syntheses of HIV-1-induced DNA, RNA and protein were minimally inhibited by rIFN α but virus particles released from the treated cells were 100- to 1000-fold less infectious, owing to an assembly defect in gp120 (Hansen *et al.*, 1992). Our published data (Krugner-Higby *et al.*, 1995) indicated that anti-HIV PC lipids do not affect HIV-1-induced protein synthesis and processing. However, assembly of progeny virus particles made in the presence of antiviral PC lipids was shifted from the plasma membrane to intracytoplasmic vacuoles (Kucera *et al.*, 1990b), and this shift in assembly site was associated with a profoundly reduced capacity of progeny virus particles to bind to CD4⁺ cells (Krugner-Higby *et al.*, 1995). Also, we observed that exposure of fresh CD4⁺ cells to equivalent amounts of RT activity associated with HIV-1 particles from 1 μ M PC lipid-treated cells resulted in a 40 to 68% inhibition in subsequent HIV-1 replication compared to HIV-1 particles from untreated control cells (LS Kucera, N Iyer, SL Morris-Natschke, SY Chen, F Gumus, K Ishaq & DBJ Herrmann, unpublished results). Data from Western blot analysis of virus particles from PC lipid-treated cells indicated that gp120 produced in infected cells was also present in virus particles recovered from PC lipid-treated cell supernatants (Krugner-Higby *et al.*, 1995). Incubation of PC lipid with preformed virions did not produce a virucidal effect (LS Kucera, N Iyer, SL Morris-Natschke, SY Chen, F Gumus, K Ishaq & DBJ Herrmann, unpublished results). Krugner-Higby *et al.* (1995) showed that HIV particles made in the presence of phospholipids had a reduced capacity to bind to CD4⁺ cells. Taken together, our data suggest that the reduced capacity of progeny virus from PC lipid-treated cells to infect and replicate in fresh CD4⁺ cells could be associated with an alteration in the assembly of gp120 on the surface of progeny virus particles. Thus, PC lipids may share a property in common with rIFN- α by operating at the stage of HIV gp120 assembly and release.

In summary, results from chemical synthesis and evaluation of compounds for anti-HIV-1 activity in this present investigation indicated that compounds with hydrocarbon chain lengths of 10 to 12 at position 1 and 8 to 12 at position 2 of a three carbon backbone have high SI against HIV-1 replication. PC lipid analogues have potent and

selective activity against HIV-1 and HIV-2 as well as against TIBO- and AZT-resistant strains of HIV-1 and virus-induced cell fusion. In view of the markedly reduced toxicity of PC lipid analogues against bone marrow cell growth *in vitro* compared to AZT, PC lipid analogues offer an innovative approach toward development of novel strategies for the treatment of HIV infections and AIDS.

References

- Chou T-C & Talalay P (1987) Applications of the median-effect principle for the assessment of low-dose risk of carcinogens and for the quantitation of synergism and antagonism of chemotherapeutic agents. In *New Avenues in Developmental Cancer Chemotherapy*, vol. 8, pp. 37-64. Bristol-Myers Cancer Symposia. Edited by K Harrap & T Connors. New York: Academic Press.
- De Clercq E (1995) Toward improved anti-HIV chemotherapy: therapeutic strategies for intervention with HIV infections. *Journal of Medicinal Chemistry* 38:2491-2517.
- Feng Y, Broder CC, Kennedy E & Berger EA (1997) HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272:872-877.
- Hansen BD, Nara PL, Maheshwari RK, Sidhu GS, Bernbaum JG, Hockema D, Meltzer MS & Gendelman HE (1992) Loss of infectivity by progeny virus from alpha interferon-treated human immunodeficiency virus type 1-infected T cells is associated with defective assembly of envelope gp120. *Journal of Virology* 66:7543-7548.
- Hansen WJ, Murai R, Wedmid Y & Baumann WJ (1982) An improved procedure for the synthesis of choline phospholipids via 2-bromoethyl dichlorophosphate. *Lipids* 17:453-459.
- Johnson VA, Walker BD, Barlow MA, Paradai TJ, Chou T-C & Hirsch MS (1989) Synergistic inhibition of human immunodeficiency virus type 1 and type 2 replication *in vitro* by castanospermine and 3'-azido-3'-deoxythymidine. *Antimicrobial Agents and Chemotherapy* 33:53-57.
- Krugner-Higby L, Goff D, Edwards T, Iyer N, Neufeld J, Kure T, Morris-Natschke S, Ishaq K, Piantadosi C & Kucera LS (1995) Membrane-interactive phospholipids inhibit HIV type 1-induced cell fusion and surface gp160/gp120 binding to monoclonal antibody. *AIDS Research and Human Retroviruses* 11:705-712.
- Kucera LS, Iyer N, Leake E, Raben A, Modest EJ, Daniel LW & Piantadosi C (1990a) Novel membrane-interactive ether lipid analogs that inhibit infectious HIV-1 production and induce defective virus formation. *AIDS Research and Human Retroviruses* 6:491-501.
- Kucera LS, Iyer N, Leake E, Raben A, Daniel L, Modest EJ & Piantadosi C (1990b) Effect of membrane-active ether lipid (EL) analogs on human immunodeficiency virus production measured by plaque assay. *Annals of the New York Academy of Sciences* 616:545-548.
- McKnight A, Wilkinson D, Simmons G, Talbot S, Picard L, Ahuja M, March M, Hoxie JA & Clapham PR (1997) Inhibition of human immunodeficiency virus fusion by a monoclonal antibody to a co-receptor (CXCR4) is both cell type and virus strain dependent. *Journal of Virology* 71:1692-1696.
- Meyer KL, Marasco CJ Jr, Morris-Natschke SL, Ishaq KS & Piantadosi C (1991) *In vitro* evaluation of phosphocholine and quaternary ammonium containing lipids as novel anti-HIV agents. *Journal of Medicinal Chemistry* 34:1377-1383.
- Morris-Natschke SL, Oquendo F, Marasco CJ Jr, Meyer KL, Marx M, Piantadosi C, Layne MD & Modest EJ (1993) Synthesis of phosphocholine and quaternary amine ether lipids and evaluation of *in vitro* antineoplastic activity. *Journal of Medicinal Chemistry* 36:2018-2025.
- Piantadosi C, Marasco CJ Jr, Morris-Natschke SL, Meyer KL, Gümüs F, Surles JR, Ishaq KS, Kucera LS, Iyer N, Wallen CA, Piantadosi S & Modest EJ (1991) Synthesis and evaluation of novel ether lipid nucleoside conjugates for anti-HIV-1 activity. *Journal of Medicinal Chemistry* 34:1408-1414.
- Ryu EK & MacCoss M (1979) Modification of the Dittmer-Lester reagent for the detection of phospholipid derivatives on thin-layer chromatograms. *Journal of Lipid Research* 20:561-563.
- Smith MS, Thresher RJ & Pagano JS (1991) Inhibition of human immunodeficiency virus type 1 morphogenesis in T cells by alpha interferon. *Antimicrobial Agents & Chemotherapy* 35:62-67.
- Storme GA, Berdel WE, van Blitterswijk WJ, Bruynel EA, DeBruyne GK & Mareel MM (1985) Anti-invasive effect of racemic 1-O-octadecyl-2-O-methylglycero-3-phosphocholine on M04 mouse fibrosarcoma cells *in vitro*. *Cancer Research* 45:351-357.
- van Blitterswijk WJ, Hilkmann H & Storme GA (1987) Accumulation of an alkyl lysophospholipid in tumor cell membranes affects membrane fluidity and tumor cell invasion. *Lipids* 22:820-823.
- White EL, Buckheit RW Jr, Ross JJ, Germany JM, Andries K, Pauwels R, Janssen PAJ, Shannon WM and Chirigos MA (1991) A TIBO derivative, R82913, is a potent inhibitor of HIV-1 reverse transcriptase with heteropolymer templates. *Antiviral Research* 16:257-266.
- Wiley RL, Bonifacino JS, Potts BJ, Martin MA & Klausner RD (1988) Biogenesis, cleavage, and degradation of the human immunodeficiency virus type 1 envelope glycoprotein gp160. *Proceedings of the National Academy of Sciences, USA* 85:9580-9584.

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